Non-Invasive Imaging of Cell Signaling

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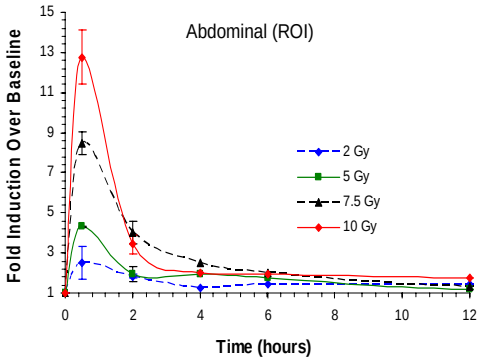
With recent advances in Genetics, Chemistry and High Throughput Screening (HTS), a large number of 'targets' and 'lead molecules' have been identified. However, this poses a tremendous challenge for selecting and/or validating these 'targets' and for broad profiling of these lead molecules against a variety of disease models for 'candidate' selection. In addition, the revolution in molecular biology has expanded our understanding of the genetics and biochemistry of transformed cells. These tremendous advances have been made largely through studies of cultured cells or *ex vivo* studies on tumor specimens. However, it is clear that extrapolations between *in vitro* and *in vivo* situations do not always hold true. Molecular imaging technologies have the potential to address these scientific and technological challenges. Non-invasive technologies like MRI, MRS, PET and Optical Imaging will contribute significantly to drug discovery research with emphasis on drug efficacy, on the mechanism of action and on target validation studies in animal disease models *in vivo*. The specific focus of current research is to develop and test the use of molecular imaging endpoints instead of time-consuming dissection and histology so as to significantly decrease the workload involved in tissue analysis and thereby speed up the evaluation of drug candidates. As imaging methods are non-invasive, they allow for longitudinal studies in a single animal. This increases the statistical relevance of a study, allows for more clinically relevant study designs and decreases the number of animals required. Imaging can also provide important information on the optimal timing and dosing of drugs. Through the development of molecular-imaging tools one can provide much earlier surrogate markers of therapy success than is currently possible.

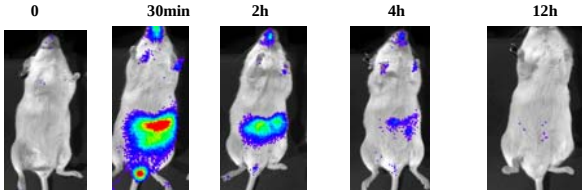
We have selected major biological phenomena and have designed creative approaches to quantitatively and non-invasively image these processes *in vivo*. Study of these biological phenomena *in vivo* using non-invasive strategies represents a major research endeavor of the Center for Molecular Imaging at Michigan. Below are brief introductions to each of these biological events, as well as a discussion of their significance in cancer. These molecular events include carcinogenesis, cellular signaling, and the apoptotic response to anti-cancer therapeutics.

Carcinogenesis

The development of tumor cells from normal cells requires the sequential acquisition of mutations to several genes. These genes fall into two major categories, the first can be referred to as oncogenes, genes that normally act to promote cell division and a second category that includes genes that function to arrest cell division, commonly referred to as tumor suppressor genes. Cancer can therefore arise from activation of oncogenes and/or inactivation of tumor suppression genes. The p53 tumor suppressor protein is unique in that it has the ability to detect the presence of damaged DNA (carcinogenesis) wherein it blocks cell cycle progression to allow for the damaged DNA to be repaired (thus ensuring that mutations aren't propagated) but it also has the ability to activate the cellular suicide program such that cells having un-repairable DNA are eliminated from the organism. This unique role of p53 as the "*Guardian of the genome*" is exemplified by the fact that 60% of all tumors carry a mutation in the p53 gene and that inherited mutations in p53 strongly predispose individuals to cancer.

We have constructed a transgenic mouse wherein the P53 responsive MDM-2 promoter driving luciferase is integrated into the genome. Therefore activation of p53 such that it now becomes transcriptionally active would result in the expression of luciferase. Expression of luciferase can then be imaged by the fact that cells having luciferase would emit bioluminescent light in the presence of luciferin (injected intraperiotenally 20 min before imaging). Emission of light from these cells can be imaged by a CCD camera. The data shown here demonstrate that p53 activation is proportional to the dose of DNA damaging radiation and that peak activation occurs at 30 mins after irradiation and is silenced within 6 hours.

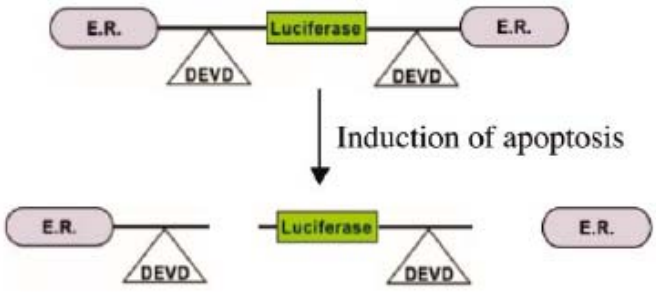




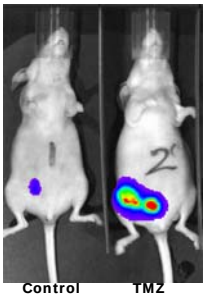
Apoptosis

Over the last decade, it has become clear that cancer is as much a disease of cell death as one of cell proliferation. The earliest demonstration of this concept was made when constitutive activation of the anti-apoptotic gene bcl-2 was shown to lead to B-cell lymphoma. These and subsequent findings have lead to the hypothesis that mutations which attenuate apoptotic responses facilitate neoplastic transformation by allowing the accumulation of other growth-promoting mutations which would otherwise commit a cell to suicide in the absence of external growth cues. It has also been proposed that tumor progression exerts a selective pressure for cells resistant to apoptosis. Evolution toward a "survivor" phenotype may be a product of the hypoxia, nutrient starvation, and falling pH that may be produced as tumor cells outgrow their blood supply. The selective pressure may be especially strong just prior to the "angiogenic switch" when dormant tumor growth is thought to be the result of balanced cellular proliferation and apoptosis. The resulting defects in the apoptotic response of tumor cells arising from early events in carcinogenesis or as a result of selective pressures are also thought to contribute to the resistance of tumor cells to cytotoxic therapies. Although the alterations of the apoptotic program are clearly important in cancer biology, a number of issues remain controversial. Numerous studies have attempted to correlate the prognosis with the apoptotic cell fraction in tumor biopsy specimens. These studies have not produced simplistic results (3). In some cases a high apoptotic fraction correlated with slower tumor growth. In other cases, however, extensive tumor cell apoptosis was either unrelated to prognosis or associated with more aggressive tumor growth. How the apoptotic index of a tumor cell population changes over tumor growth also remains unclear. The relative contributions of apoptosis and necrosis to radiation and drug-induced cell death also remains controversial. Changes in apoptotic fractions following acquisition of the angiogenic phenotype are also not well characterized.

Currently available techniques for studying apoptosis in solid tumors make it difficult to study these problems. Scoring apoptotic indices by morphological criteria is time consuming and requires skilled observers. Specific staining of apoptotic cells, such as the TUNEL method for marking the 3' termini of cleaved DNA, is also time consuming and may have a significant false-positive rate. The ability to non-invasively image apoptosis in live animals dynamically over time would significantly enhance our understanding of the role of apoptosis in neoplastic transformation as in the response of tumors to therapies.



The strategy for imaging of apoptosis. A Chimeric polypeptide consisting of a reporter molecule (luciferase) fused to the estrogen receptor regulatory domains (ER) resulted in the most efficient silencing of the reporter activity. Inclusion of a protease cleavage site between these domains provided for protease-mediated activation of the reporter molecule after release of the silencing domains (i.e., ER). For example, inclusion of the DEVD sequence (a caspase-3 cleavage site) between ER and Luc results in the release of Luc from the silencing domain from the amino and the carboxyl termini in a caspase-3-dependent manner. Because most cells activate caspase-3 during apoptosis, this reporter construct can be used for reporting (imaging) of apoptosis.



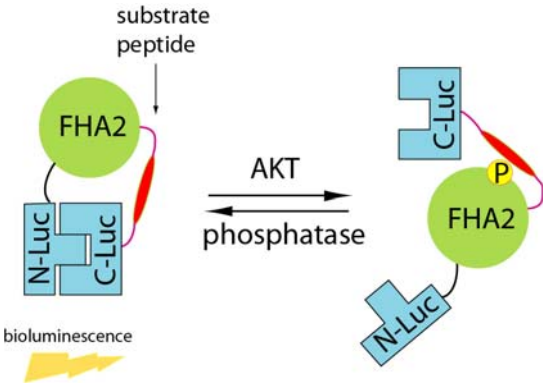
Imaging of apoptosis within tumors in response to chemotherapy. Nude mice bearing human glioma xenograft tumors (D54) expressing the above reporter molecule were treated with Temozolamide. Low levels of light were detected in control, untreated animals while Temozolamide treated animals had a significant increase in bioluminescence activity after treatment.

Imaging of Akt signaling

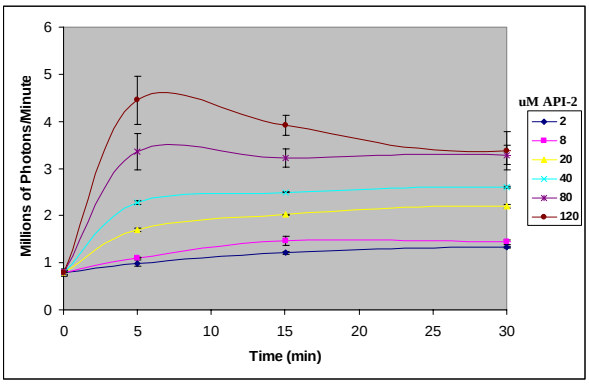
The serine/threonine kinase Akt functions as a signaling hub wherein many upstream signaling pathways (involving stimulation of receptor tyrosine kinases (RTK) such as IGF-1R, HER2/Neu, VEGF-R, PDGF-R) converge. The integration of these intracellular signals at the level of Akt and its kinase activity, regulates the phosphorylation of several downstream effectors, such as NF-kappa B, mTOR, Forkhead, Bad, GSK-3 and MDM-2. These phosphorylation events in turn mediate the effects of Akt on cell growth, proliferation, protection from pro-apoptotic stimuli, and stimulation of neo-angiogenesis. Because Akt and its upstream regulators are deregulated in a wide range of solid tumors and hematologic malignancies, and in view of the aforementioned biologic sequelae of this pathway, the Akt pathway is considered a key determinant of biologic aggressiveness of tumors, and a major potential target for novel anti-cancer therapies. Ongoing efforts have focused on therapeutically targeting Akt and its

biologic sequelae, either at the level of Akt itself or at the levels of its upstream regulators and downstream effectors. Because Akt is also important for proliferative and anti-apoptotic signaling pathways critical for normal cells, particular emphasis is placed on the fine-tuning the targeting of individual components of this pathway to maximize the therapeutic index of anti-cancer strategies based on the PI-3K/Akt pathway.

To facilitate *in vivo* studies wherein the efficacy of targeted therapeutic agents is optimized, non-invasive monitoring of Akt function will prove to be a beneficial tool. To this end we here describe the development of a recombinant molecule that non-invasively reports on Akt activity in living cells and animals. This reporter contains an Akt consensus substrate peptide and a cognate phosphor-Serine/Threonine binding domain (FHA2 domain of Rad53). At either ends of this fusion the two halves of luciferase are tethered together by a linker.



Imaging of Akt function using the AktpepFHA2 luciferase reporter. The reporter molecule is constitutively active in the absence of Akt activity. Phosphorylation of the Akt-peptide in response to Akt activity should result in association of FHA2 to the Akt phospho-peptide, thus resulting separation of N-Luc and C-Luc which will therefore result in loss of bioluminescence activity.



Non-Invasive imaging of Akt function using bioluminescence imaging: A stable cell line using D54 glioma cells (having constitutively active Akt) was constructed to express the AktpepFHA2 reporter. Since the cell line has active Akt, low levels of light are detected from the reporter cell line. Inhibition of Akt using API-2 (Tocris, CA) results in a time and dose dependent increase in bioluminescence activity. These results collected using non-invasive methods correlated with invasive assays of Akt status (western blot analysis) thus validating the reporter molecule as a specific and quantitative reporter of Akt status.